Annals of Clinical and Analytical Medicine
Original Research

Effect of different latency periods on distraction osteogenesis in an experimental rabbit model

Distraction osteogenesis and latent period

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Abstract

Aim: This work used immunohistochemical and radiographic analysis to show how latency time affects distraction osteogenesis in animals.

Material and Methods: Eighteen rabbits had right femur drill osteotomies and external fixators. Latency duration split the rabbits into three groups. Groups 1, 2, and 3 had latency times of 1, 5, and 10 days. After the latency period, all groups had a 20-day consolidation phase. After consolidation, the animals were euthanized, and immunohistochemical, histological, and radiological studies were performed on the distraction-induced callus tissue.

Results: In radiological evaluation, there was no significant difference between Group 1 and Group 2 and Group 2 and Group 3 (p=1,000, p=0,066). Group 1 and 3 differed (p=0.018). Histopathological assessment showed no significant difference between Group 1 and Group 2 or Group 2 and Group 3 (p= 0,557, p=0,062). Group 1 and 3 differed (p=0,001). Group 3 had more osteocalcin-positive cells than Groups 1 and 2.

Discussion: Histopathological and radiographic methods showed that a 10-day distraction osteogenesis latency time in rabbit femurs is safer than 1 and 5-day periods. Human distraction osteogenesis latency must be determined by clinical trials.

Keywords

Distraction Osteogenesis, Latency Period, Osteotomy, Rabbit Model

DOI: 10.4328/ACAM.21887 Received: 2023-08-19 Accepted: 2023-09-26 Published Online: 2023-10-14 Printed: 2023-10-15 Ann Clin Anal Med 2023;14(Suppl 3):S248-253
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This study was approved by the Ethics Committee of Istanbul Mehmet Akif Ersoy Experimental Research And Development Center (Date: 2017-03-30, No: 2017/04)

Introduction

Distraction osteogenesis (DO), also known as callus distraction, is a commonly used method for treating bone loss, pseudoarthrosis, chronic osteomyelitis, leg length inequality, and deformities. It involves the biological production of new bone and accompanying soft tissues by gradually applying traction force to surgically separated bone segments using an external fixator. The process of DO consists of three stages: latency, distraction, and consolidation periods. Various factors, such as the length of the latency period, duration of the consolidation phase, distraction rate and rhythm, the type or level of osteotomy, and stability of fixation, influence the success of DO. However, the optimal length of the latency time, ranging from 3 to 10 days, remains a topic of debate and varies based on individual surgeons' experiences [1,2]. In this study, we evaluated the quality of ossification in the distraction regions of rabbit femurs using histopathological, immunohistochemical, and radiological techniques, comparing different latency times (1, 5, or 10 days). Our goal was to contribute to the existing literature by filling the gap regarding the optimal latency time. We hypothesized that the experimental 10-day latency period would yield superior results compared to shorter latency times in DO, as demonstrated through an animal model.

Material and Methods

Experimental Groups

All animals used in the study were ethically treated in accordance with the Laboratory Animal Care and Use Guidelines. Eighteen New Zealand rabbits (male, twenty-month-old, 2.8-3 kg) were randomly assigned to three groups based on latency duration. Six animals per group. Latency periods were 1, 5, and 10 days for Groups 1, 2, and 3. Distraction began after the latency intervals. Mini sliding monolateral finger external fixators (Tasarimmed, Istanbul, Turkey) were mounted to the rabbits' right femurs, and numerous drill osteotomies were done. For 10 days, 1.0 mm/day was distracted. For consolidation, the rabbits wore the external fixator for 20 days after distraction. After surgery, rabbits had cage freedom. Propofol intracardiac injection euthanized the rabbits after consolidation. X-rays of the femurs were obtained at equal dosages and 48 cm apart. After dissection, the extended femurs were photographed. Bone slices, including a 10 mm segment from the distracted region, were cut with a bone saw (0-1200 rpm, Syntes) and deposited in 10% neutral formalin for histological evaluation.

Istanbul Mehmet Akif Ersoy Experimental Research and Development Center surgeon performed all surgeries under sterile settings. The Animal Ethics Committee at Istanbul Mehmet Akif Ersoy Experimental Research and Development Center granted ethical approval (2017/04). One dosage of 25 mg/kg cefazolin sodium (Cefazol, Mustafa Nevzat, Turkey, i.m.) was given preoperatively. The rabbits were weighed and anesthetized with 7 mg/kg Xylazine hydrochloride (Rompun, Bayer, 23.32 mg/ml, i.m.) and 35 mg/kg Ketalar (Pfizer, 50 mg/ml).

After anesthesia, the right thigh was shaved and prepped with 10% povidone-iodine solution. The proximal and distal right femur were bored with a 1 mm drill and fastened with a tissue protector using 2 mm Schanz screws under sterile conditions. Then, mini sliding monolateral finger external fixator systems (Tasarimmed, Istanbul, Turkey) were applied with proximal and distal Schanz screws. The monolateral external fixator system has two Schanz screws at the proximal and distal ends.

After installing the fixator system, an 11 mm scalpel made a 1 cm incision in the lateral diaphysis of the femur. The fascia was longitudinally cut by 1 cm to access the bone through the vastus lateralis-tensor muscle cleavage. Two micro Hohmann retractors exposed the osteotomy line without periosteum injury. Multiple drill osteotomy was used to transversely osteotomize the femoral diaphysis. The ability to remove 1 mm of the osteotomy line confirmed completion. The fascia and dermis were sutured with 5/0 fast vicryl, and the incision and Schanz screws were dressed.

Animals were euthanized after consolidation. Xylazine hydrochloride (Rompun, Bayer, 23.32mg/ml) was intramuscularly delivered as a pre-anesthetic, followed by intracardiac propofol to produce anesthesia and euthanasia. The right femurs of deceased animals were carefully dissected to remove surrounding muscles, and a bone portion (Figure 1), including the distracted area, was transversely cut using a low-speed vibrating bone saw (12,000 vibrations/min, Synthes). The removed bone tissues were put in 10% neutral formalin.

Radiological Evaluation

Radiographs of each rabbit were acquired at the Istanbul Mehmet Akif Ersoy Experimental Research and Development Center. Anteroposterior radiographs (Figure 2) and lateral radiographs of the surgically treated right femurs were obtained using a faxitron device. The radiographs were taken at equal doses from a distance of 48 cm. Subsequently, the radiographs were transferred to a computer with an interface for evaluation, following the modified Lane and Sandhu radiological scoring system (Table 1) [3].

Histopathological and Immunohistochemical Analyses

The collected bone samples were fixed in 10% neutral formalin and then subjected to decalcification. After the decalcification process, the samples were washed with tap water. Secondary fixation was performed using 10% neutral formalin, following the routine tissue fixation procedure. The tissues were dehydrated using increasing concentrations of ethanol, permeabilized with toluene, and embedded in paraffin. Transverse sections, approximately 5 µm thick, were obtained from the paraffin blocks using a microtome (Leica). The sections were deparaffinized and rehydrated, and then stained with hematoxylin and eosin (H&E) and Masson's trichrome. These stains were used to examine the newly formed bone trabeculae, fibrous tissue, and chondral tissue areas. The sections were analyzed using an Olympus BX61 microscope, and images were captured with an Olympus DP72 camera. To evaluate the sections, the histological scoring system developed by Lane and Sandhu, and modified by Heiple et al. was employed (Table 1) [4].

For immunohistochemistry, the deparaffinized and rehydrated sections were subjected to antigen retrieval using 1/10 EDTA buffer. To block endogenous peroxidase activity, 3% hydrogen peroxide was applied. After blocking, the slides were incubated with primary antibodies, including osteocalcin (Abcam) and CD34 (Abcam), for two hours in a humidity chamber at room temperature. Amplifier Quanto and HRP Polymer Quanto (both

from Thermo Scientific) were sequentially applied to the slides. The detection of osteocalcin and CD34 positive cells was achieved using DAP substrate. Harris Hematoxylin was used as a counterstain. Sections were examined using an Olympus BX61 microscope, and images were captured with an Olympus DP72 camera.

Statistical Analysis

The power analysis of the study, "G. Calculated using the "Power-3.1.9.2" program. As a result of the analysis applied to 18 animals, the effect size was found to be 2,2456 at the level of α=0,05 and the power of the study, which was calculated as post-hoc, was calculated as 1,00. The minimum required power value for a post-hoc analysis is 0,67. In this case, the power analysis is at an acceptable level, the number of data is sufficient.

The data obtained in the research were analyzed using the SPSS (Statistical Package for Social Sciences) 25.0 for Windows program. While evaluating the data, descriptive statistical methods (min-max values, median, mean, standard deviation) were used. It was observed that the data used did not fit the normal distribution, since the skewness and kurtosis values were not between ±1 and the Shapiro-Wilk test p value was ≤ 0,05. The Kruskal-Wallis test was used to evaluate the differences between the three independent groups, and if there was a difference, the Bonferroni correction was used to find the group that made a difference. P- value ≤ 0,05 was considered statistically significant.

Ethical Approval

Ethics Committee approval for the study was obtained.

Results

Radiological Results

Radiographs of the rabbit right femurs revealed limited ossification in the distraction areas of Groups 1 and 2, whereas nearly complete ossification was observed in Group 3. These results were further supported by a semi-quantitative scoring system based on the Modified Lane and Sandhu radiological scoring system. After statistical analysis, a statistically significant difference was found between these three groups (Kruskal-Wallis test, p=0.013). In radiological evaluation, as a result of the pairwise comparison of the groups, there was no significant difference between Group 1 and group 2, and between group 2 and group 3 (p= 1,000, p=0,066). A significant difference was observed between group 1 and group 3

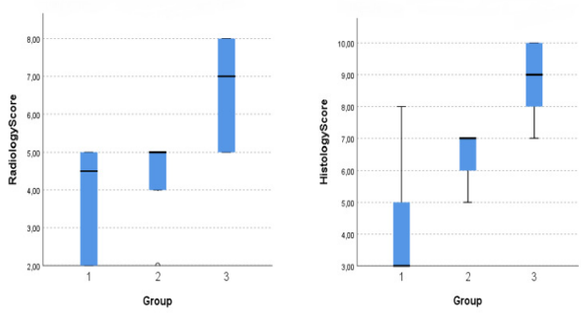

Figure 1. Statistical Analysis Graph of Radiological and Histological Scores.

Table 1. Modified Lane and Sandhu radiologic scoring system and Lane and Sandhu scoring system modified by Heiple et al.

Lane and Sandhu histology scoring system		Modified Lane ve Sandhu radiologic scoring system	
Union		Bone formation	
No evidence of union	0	No evidence of bone formation	0
Fibrous union	1	Bone formation occupying 25% of defect	1
Osteochondral union	2	Bone formation occupying 50% of defect	2
Bone union	3	Bone formation occupying 75% of defect	3
Complete organization of shaft	4	Full gap bone formation	4
Cancellous bone		Union	
No osseous cellular activity	0	Nonunion	0
Early apposition of new bone	1	Possible union	1
Active apposition of new bone	2	Radiographic union	2
Reorganizing cancellous bone	3	Remodeling	
Completely reorganizing cancellous bone	4	No evidence of remodeling	0
Cortical bone		Remodeling of medullary canal	1
None	0	Full remodeling of cortex	2
Early appearance	1	Total point possible per category	
Formation under way	2	Bone formation	4
Mostly reorganized	3	Union	2
Completely formed	4	Remodeling	2
Marrow		Maximum Score	8
None in resected area	0		
Beginning to appear	1		
Present in more than half of the defect	2		
Complete colonization by red marrow	3		
Mature fatty marrow	4		
Total points possible per category			
Union	4		
Cancellous bone	4		
Cortex	4		
Marrow	4		
Maximum score	16		

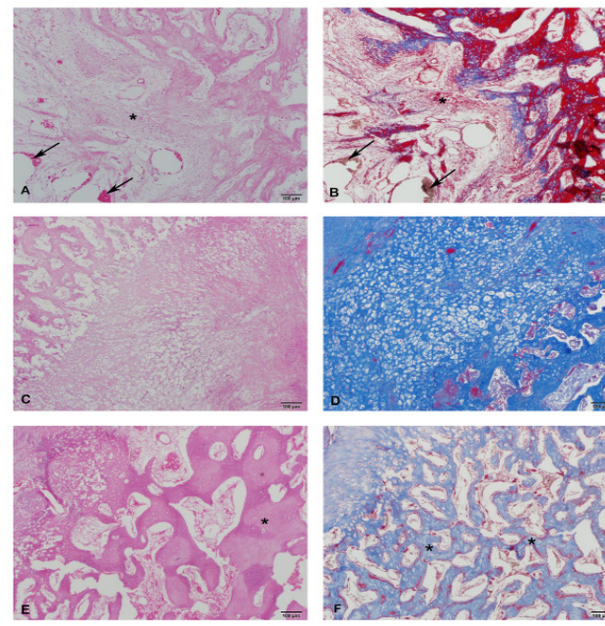

Figure 2. a and b Fibrosis (*), which indicate intramembranous ossification and blood vessels (arrows) associated with hemorrhage were distinct in the 1st group. c and d endochondral ossification zones and newly formed bone spicules were seen in the 2nd.

Table 2. Radiologic and histologic scores and statistical analyses of scores by groups.

	Group 1		Group 2		Group 3	
	Mean ± SD	Min Max Med	Mean ± SD	Min Max Med	Mean ± SD	Min Max Med
Radiology Score	3,83 ± 1,47	20 50 4,50	4,33 ± 1,21	2,00 5,00 5,00	6,66 ± 1,36	5,00 8,00 7,00
Histology Score	4,00 ± 2,00	3,00 8,00 3,00	6,83 ± 0,41	6,00 7,00 7,00	9,50 ± 0,55	9,00 10,00 9,50
Union	1,17 ± 0,41	1,00 2,00 1,00	1,83 ± 0,41	1,00 2,00 2,00	2,00 ± 0,00	2,00 2,00 2,00
Spongious Bone	1,33 ± 0,52	1,00 2,00 1,00	2,00 ± 0,00	2,00 2,00 2,00	3,00 ± 0,00	3 3 3
Cortical Bone	1,17 ± 0,41	1,00 2,00 1,00	2,00 ± 0,00	2,00 2,00 2,00	2,83 ± 0,41	2,00 3,00 3,00
Bone Marrow	0,33 ± 0,82	0,00 2,00 0,00	1,00 ± 0,00	1,00 1,00 1,00	1,67 ± 0,52	1,00 2,00 2,00

	Kruskal-Wallis Test (between three groups) p-value	Pairwise Comparisons by the Bonferroni Correction		
		Group 1 and 2 p-value	Group 1 and 3 p-value	Group 2 and 3 p-value
Radiology Score	0,013	1	0,018	0,066
Histology Score	0,001	0,557	0,001	0,062
Union	0,007	0,052	0,09	1
Spongious Bone	0,001	0,049	0	0,044
Cortical Bone	0,001	0,18	0,001	0,18
Bone Marrow	0,011	0,397	0,008	0,397

SD: Standard Deviation; Min: Minumum Max: Maximum; Med: Median

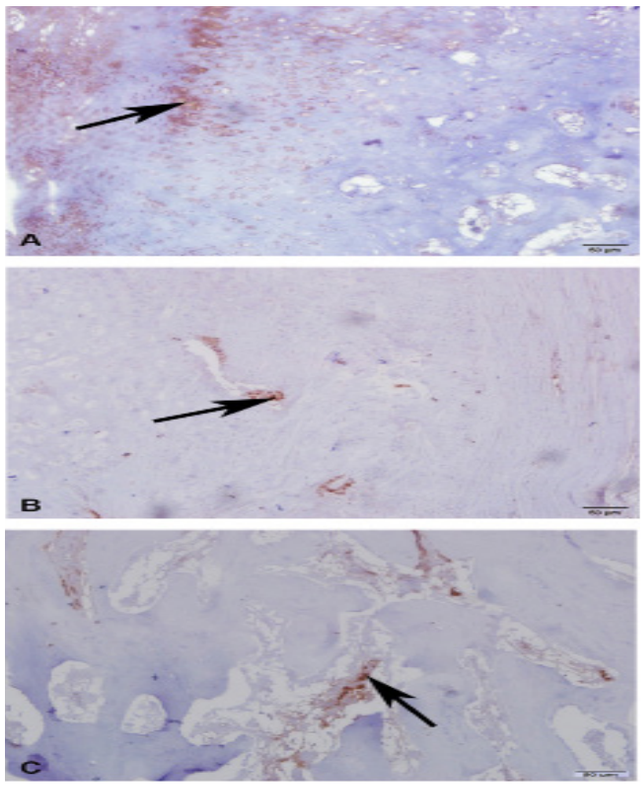

Figure 3. a Osteopontin, b osteocalcin, c CD34 immunoreactive cells (arrows) Bar: 50 µm.

(p=0,018). Detailed radiological and histopathological analysis results are presented in Table 2 and Figure 1.

Histological and immunohistochemical results

Group 1 predominantly showed fibrous tissue in the distraction area, while group 2 exhibited the presence of newly formed bone spicules, indicating intramembranous ossification. Group 3 showed a combination of hyaline cartilage and large bone trabeculae, which can be interpreted as endochondral ossification (Figure 2). After comparison of the groups using the modified Lane and Sandhu histological scoring system, a statistically significant difference was found between these three groups (Kruskal-Wallis test, p=0,001). As a result of the pairwise comparison of the groups, there was no significant difference between group 1 and group 2, and between group 2 and group 3 (p= 0.557 , p=0.062), but a significant difference was observed between group 1 and group 3 (p=0.001) (Table 2). Immunohistochemical analysis demonstrated osteocalcin immunoreactivity in osteoblasts surrounding the newly formed bone matrix in group 1. In group 2, osteocalcin immunoreactivity was also observed in hematopoietic cells. Group 3 exhibited relatively higher levels of osteocalcin immunoreactivity compared to group 1 and group 2, and CD34 positivity was not detected in group 3 (Figure 3).

Discussion

Distraction osteogenesis promotes bone repair after osteotomy by controlled separation of vascularized bone segments. Distraction osteogenesis is commonly employed to regenerate bone following osteotomy, however the latency period is not well-defined [5]. Immediate distraction may improve outcomes [6-8]. Thus, this work investigated the optimal distraction osteogenesis latency duration in the rabbit femoral diaphysis utilizing multiple drill osteotomy and a monolateral fixator. This study found that group 3, with a 10-day delay time, had superior distracted zone ossification than groups 1 and 5. Histological and radiological investigations indicated that groups with latency durations of 1 to 5 days had fibrous tissue at distraction sites and poor recovery. The 10-day latency zone had considerable bone trabeculae development.

Aida et al. examined latency period in distraction osteogenesis (DO) of the craniomaxillofacial skeleton in rabbits [1]. Their findings showed that 0 and 2 days were too short to generate bone trabeculae, while 10 days were too long and caused early consolidation. A distraction zone with immature bone trabeculae formed after 5 days. These findings show that rabbit mandibular DO may benefit from a 5-day latency period. Our study showed that bone growth was better after 10 days than 1 or 5 days. Distracted bone types may explain this variance. The mandible is porous and has better blood flow than the compact femur, which may shorten latency.

Distraction length and frequency encourage bone growth mechanically. Ilizarov, a leading expert, stressed the significance of harmonizing these aspects to avoid problems. Ilizarov states that a daily distraction of 4 x 0.125 mm (0.5 mm per day) causes excessive osteogenesis and premature consolidation, whereas 4 x 0.5 mm (2 mm per day) causes poor bone development and soft tissue injury [9]. Ilizarov indicated that a daily distraction of 4 x 0.25 mm optimizes osteogenesis. Animal studies demonstrate

that once-daily distraction works [10,11]. Since our investigation was on animals, we applied a 1 mm distraction daily.

Distraction osteogenesis also depends on consolidation time. Consolidation is usually characterized as twice the distraction time [12]. Some research recommends 28-36 days [13]. Our consolidation period was initially 20 days, but it was too short for total healing. Our data suggest that the consolidation phase should be double the distraction time for optimal results.

DO ossification type is debated. A rabbit limb lengthening model revealed intramembranous ossification of distracted bone callus tissue [14]. Ilizarov claimed that under optimal fixation conditions with a "tension-stress effect," new bone creation occurs directly from fibrous tissue without a cartilage layer, mimicking intramembranous bone development [9]. However, following dog and sheep DO models showed intramembranous and endochondral ossifications [15,16]. Paley et al. found that intramembranous ossification is the main method of bone production, except in cases of vascular damage caused by rapid distraction and unstable fixation [17,18]. Fixator stability affects distraction osteogenesis ossification. Micro-movements between bone fragments can impair consolidation, fibrocartilage callus development, and local circulation. Thus, fixation is critical during the operation. Circular and unilateral external fixators are utilized [15]. Our investigation used monolateral fixation, which is less stable than circular fixation but resists bending and torsional stresses better. The difficulty of surrounding rabbit femurs and the requirement to preserve their mobility influenced the selection [15,19]. Due to diminished stability, group 1 showed fibrosis, the early stage of intramembranous ossification, while groups 2 and 3 showed endochondral ossification in the distraction zones.

Osteocalcin, generated by osteoblasts, regulates bone mineralization [20,21]. Osteoprogenitor cells also express CD34 [22,23]. In our work, distraction zone slices were immunohistochemically analyzed for osteocalcin and CD34. The 10-day delay group had more osteocalcin-positive cells than the 1- and 5-day groups. The 10-day latency group appears to have more ossification. The 5-day latency group had CD34-positive cells, while the 10-day latency group did not. These data show that progenitor cells can develop into osteoblasts in the 10-day latency phase, whereas CD34-positive cells persist in the 5-day latency period.

Our histological and radiological findings showed that a 10-day latency time was safer than 1 and 5-day latency periods for distraction osteogenesis in rabbit femurs. Age, periosteum integrity following osteotomy, and site stability during distraction can hinder distraction osteogenesis standardization. Human distraction osteogenesis latency needs further clinical research.

Limitation

Our study is limited by a small sample size and no biomechanical evaluation. Radiographs instead of tomography may have hampered our radiological assessment. Given that all groups have callus tissue, these constraints are minimal.

Acknowledgment

We acknowledge all the researchers who have determined the effect of latency time on distraction osteogenesis in this experimental model.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and Human Rights Statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Funding: *Gaziosmanpasa Taksim Health Training and Educational Hospital, Scientific Research Project (BAP) Council (Project approval number/date: 10/14.09.2017).*

Conflict of Interest

The authors declare that there is no conflict of interest.

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How to cite this article:
Mehmet Kılıç, Ahmet Yurteri, Sami Sökücü, Mehmet Ümit Çetin, Serbay Ozkan, Engin Cetin, Gözde Erkanlı Sentürk, Ufuk Ozkaya. *Effect of different latency periods on distraction osteogenesis in an experimental rabbit model. Ann Clin Anal Med 2023;14(Suppl 3):S248-253*

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